Developmental and morphological regulation of clathrin-mediated endocytosis in *Trypanosoma brucei*

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SUMMARY

Essentially all macromolecular communication between *Trypanosoma brucei* and its host is confined to vesicular trafficking events occurring at or around the flagellar pocket. The vertebrate stage bloodstream form trypomastigote exhibits an extremely high rate of endocytosis required for nutrient uptake and probably also evasion of the host immune system. However, the rate of endocytosis is very low in the procyclic vector parasite, indicating that endocytosis is subject to a marked level of developmental regulation. Previous ultrastructural studies and crude biochemical fractionations have indicated the presence of coated pits and vesicles that are analogous to clathrin coats in the bloodstream form, but not in the procyclic. However, a definitive description of the components of this coat and its molecular function in *T. brucei* has remained elusive. We describe the molecular cloning and initial characterisation of components of the *T. brucei* endocytic coats: clathrin heavy chain (TbCLH) and a β-adaptin (TbAPβ1). TbCLH is markedly upregulated in the bloodstream form compared with the procyclic, whereas TbAPβ1 is subject to more limited developmental regulation. We generated antisera against both proteins and show that the clathrin coat is tightly associated with the flagellar pocket in both major life stages. However, in bloodstream parasites TbCLH is also extensively distributed throughout the posterior end of the cell on numerous large vesicular and tubular structures. By cryo-immuno EM, clathrin is localised to collecting tubules at the flagellar pocket and is also associated with the trans-Golgi network. These EM data confirm that the electron dense coats reported on trypanosome vesicles and tubules contain clathrin. The TbAPβ1 exhibits an atypical distribution relative to previously characterised adaptins, associating not only with the trans-Golgi but also with other tubular-vesicular elements. Localisation of TbAPβ1 is also subject to developmental regulation. These data describe major endocytic coat proteins in *T. brucei* for the first time, and indicate stage-specific expression of the clathrin heavy chain. Modulation of clathrin expression is likely to be an important factor in the developmental regulation of endocytosis and recycling in the African trypanosome.

Key words: VSG, Clathrin, Trypanosome, Endocytosis, Endosome, Adaptin

INTRODUCTION

As a deeply divergent lineage of the eukaryotes, trypanosomatids have evolved numerous unique features in gene expression and cell biology. The architypic trypanosomatid cell is a spindle-shaped structure, supported by an extensive subpellicular tubulin cytoskeleton and, for the most part, the intracellular organelles are highly spatially organised. Of particular interest in these organisms is the endocytic system, which has been implicated as having a role in virulence as well as general cellular function. The endocytic apparatus is a highly polarised system; specifically, macromolecule uptake is limited to the flagellar pocket, a small invagination in the plasma membrane where the flagellum emerges from the cell. Although this region occupies only 2-3% of the total plasma membrane (Webster and Russell, 1993), the rate of endocytosis measured in the mammalian life cycle stage of *Trypanosoma brucei* is higher than that observed in any other eukaryote, being equivalent to the internalization of the entire flagellar pocket membrane every 1-2 minutes (Coppens et. al., 1988, Webster and Griffiths, 1994) and hence represents a particularly challenging sorting problem.

T. brucei is the causative agent of sleeping sickness in humans and Nagana in cattle, a major cause of morbidity and economic hardship in endemic regions of Africa. The life cycle of *T. brucei* alternates between the mammalian bloodstream form (BSF) and tsetse fly vector procyclic form (PCF), each stage possessing a unique surface coat. As the parasite:host interface, the plasma membrane is clearly an important immune system target. To survive in the mammalian host the parasite exploits antigenic variation of the variant surface glycoproteins (VSG), which comprise the bloodstream form coat, to avoid recognition by the humoral immune response. Recent evidence has shown that during infection of rabbits, specific IgM and IgG anti-VSG antibodies are produced more rapidly than the average VSG switch, indicating that the major variant trypanosomes must be able to survive, at least temporarily, in the presence of specific VSG antibodies (O'Beirne et. al., 1998). Such antibodies cause aggregation of BSF *T. brucei* in immune serum in vitro, but metabolically

active parasites disaggregate after continued incubation and remain fully infective. Disaggregation is dependent on normal endocytic activity and, critically, results in proteolysis of internalised immunoglobulin (O'Beirne et al., 1998), suggesting that the endocytic/exocytic cycle plays a role in removal of coat-associated anti-VSG antibodies from the parasite surface, contributing to parasite defence against the host.

Clathrin-mediated vesicular traffic is a major mechanism by which proteins and lipids are transported between membrane-bound organelles and is responsible for a large proportion of import from the plasma membrane (endocytosis) and transport from the trans-Golgi network (TGN) towards the endosomal system (reviewed by Kirchhausen, 2000). Clathrin may also be involved in carrying protein and lipid from the TGN to vacuoles and lysosomes (Hirst and Robinson, 1998). Cytoplasmic clathrin is a trimer (termed a triskelion) of three heavy chains of 190 kDa radiating from a central hub; each of the heavy chains binds a ~25 kDa light chain (Ungewickell and Branton, 1981; Crowther and Pearse, 1981; Kirchhausen and Harrison, 1981). When the triskelions associate with the membrane, they assemble into a planer lattice of hexagons (Crowther and Pearse, 1981). The conversion of this lattice to one of hexagons and pentagons provides the driving force for local deformation of the membrane and formation of clathrin-coated pits and vesicles (Kirchhausen, 2000).

During the process of clathrin-coated vesicle formation, clathrin interacts with a network of protein partners in a coordinated manner. Adaptor proteins (APs) are involved in the recruitment of cargo, whereas others such as AP180, auxilin, amphiphysin, eps15 and epsin have regulatory functions (Kirchhausen, 1999). The most abundant proteins in the clathrin coat, after clathrin itself, are the heterotetrameric AP complexes, which are present at a ratio of about one per two triskelions. Several distinct, but closely related, classes of multimeric adaptors have been identified in mammals. The best characterised are the AP-1 complex specific for traffic from the TGN to the endosome and the AP-2 complex specific for traffic from the plasma membrane to the endosome. Each complex contains two large ~100 kDa subunits (β1- and γ-adaptin for AP-1, and β2 and α-adaptin for AP-2), a medium ~50 kDa subunit (μ1 or μ2) and a small ~20 kDa subunit (σ1 or σ2). Other adaptor protein complexes AP-3 (γ3, β3, μ3, σ3) found near endosomes (Dell'Angelica et al., 1997; Simpson et al., 1997) and AP-4 (ε, β4, μ4, σ4) found close to the TGN (Dell'Angelica et al., 1999; Hirst et al., 1999) have been identified but have not yet been unambiguously shown to associate with clathrin. The analogous subunit complexes are structurally related and probably fulfil similar functions; for example, the μ-subunits are involved in the recognition of tyrosine-based sorting signals (Heuser and Keen, 1988) and the β-subunits interact with cargo carrying the LL motif (Greenberg et al., 1997; Rapaport et al., 1998) and with clathrin heavy chain (Shih et al., 1995; Owen et al., 2000).

We have identified through database searches EST and whole genome shotgun sequences for several potential components of clathrin-coated vesicles in *T. brucei*. Here we report on two of these, TbAPβ1, a trypanosomal β-adaptin homologous to yeast β1-adaptin, and TbCLH, clathrin heavy chain. Both proteins exhibit differential localisation between life stages and the clathrin heavy chain is subject to marked upregulation in the BSF, suggesting clathrin expression levels correlate with the elevated endocytosis and recycling essential to the survival of the BSF parasite.

MATERIALS AND METHODS

PCF and BSF cells, strain 427, were maintained in SDM79 and HMI9 media, respectively, as described (Field and Field, 1997).

DNA cloning and manipulation

The complete open reading frame of the *T. brucei* clathrin heavy chain gene, *TbCLH*, was derived from a combination of GSS end sequences from the *T. brucei* genome project and PCR fragments amplified from *T. brucei brucei* genomic DNA to fill in gaps in the contig. BLAST searching of the TIGR *T. brucei* genome project database (www.tigr.org/tdb/mdb/tbdb) identified nine sheared genomic DNA sequences and one BAC clone end sequence with homology to several known clathrin heavy chain genes (Fig. 1A). Based on these sequences the following primers were designed to generate PCR fragments to complete the open reading frame, TbCLH1: 5′ GGGGAATTCGCTGGCGCCCCTCTGTGAAC 3′; TbCLH2: 5′ GGGAAGCTTGAACCGCGCAAATAGCAGCAA 3′; TbCLH3: 5′ GGGAAGCTTGCGAAGGGCCAGATCAGC 3′; TbCLH5: 5′ GCCATACCTCGAATCCGCAC 3′; TbCHC6: 5′ GGCCTGCAGCCTGAGTTTGACGCCTCG 3′ TbCLH7: 5′ GCCACCACACATCCGTAACAAATGG3′. The amplified fragments were subcloned using the PCR-Script Amp cloning kit (Stratagene, USA) and sequenced. The final sequences were assembled with the GSS sequences to complete the TbCLH open reading frame.

During a sequencing screen for components of the trypanosomal secretory pathway we isolated a partial cDNA identical to an EST fragment (GenBank accession no. AI881056) homologous to higher eukaryotic β-adaptins. This clone was used as a probe to screen a *T. brucei* λFIX genomic library by filter hybridisation to obtain the entire *TbAPβ1* ORF.

Northern blot hybridisation

Total RNA was extracted from mid-logarithmic phase cultures of *T. brucei* PCF and BSF cells using TrizolReagent (Gibco Life Technologies Ltd, Paisley, UK). 20 μg of RNA was separated on a 1.2% agarose/formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Life Science Ltd, UK). Hybridisation was performed in Church buffer and the filter was washed in 0.2× SSC/0.1% SDS at 65°C.

Antibody production

Antiserum against TbAPβ1 peptide (residues 568-582, CVESTFSDAMTMGDL) was generated in rabbits and mice using standard procedures. TbCLH antiserum was generated against an expressed clathrin fragment (residues 1268-1465) amplified using the following primers TbCLHF1: 5′ CGGGATCCGATGCGGTTAACCATG 3′; TbCLHR1: 5′ CCCAAGCTTGGATTCGAGGTATGGTATGGCAGAATC 3′ which was subcloned into the pQE30 expression vector (Qiagen, Max-Volmer-Strabe, Hilgen) through a *Bam*HI site introduced the 5′ primer and a *Hind*III site in the 3′ primer. Antibodies were affinity purified on antigen immobilised on cyanogen bromide activated Sepharose 4B (Pharmacia, St Albans, UK).

Immunofluorescence microscopy

Cells were washed in 250 mM Hepes pH 7.5 and, applied to poly-lysine-coated slides and fixed in 4% paraformaldehyde (PFA) in 250 mM Hepes pH 7.5 for 10 minutes on ice, followed by 8% PFA-Hepes for 10 minutes on ice and then 40 minutes at room temperature. All further manipulations were performed at room temperature.

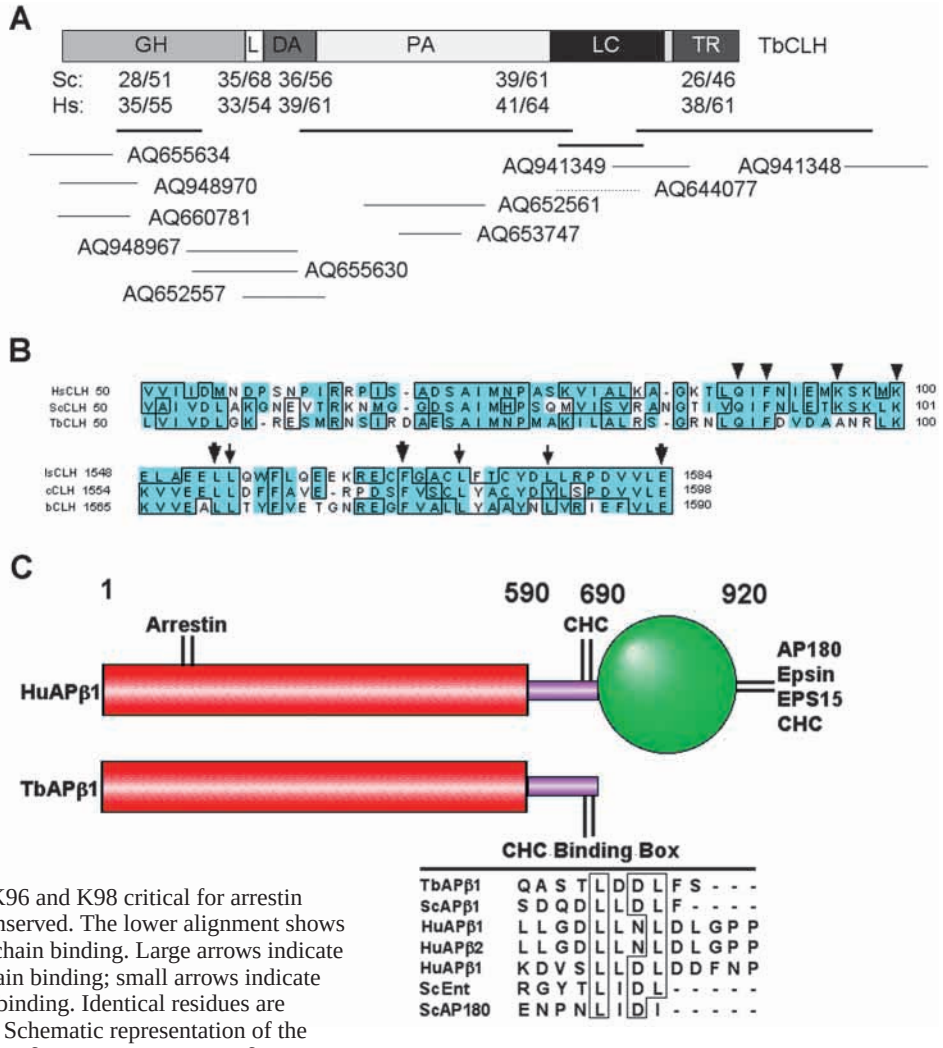

Fig. 1. Sequence features of trypanosome clathrin and adaptin. (A) The clathrin heavy chain. Structural domains of *T. brucei* (TbCLH) are depicted as determined on the basis of similarity to human CLH1 (accession no. Q00610) and yeast CLH (accession no. P22137). GH, globular head; L, flexible linker; DA, distal arm; PA, proximal arm; LC, light chain binding region; TR, trimerisation region. Percentage identity/similarity to yeast CLH (Sc) and human CLH1 (Hs) are given below each domain. The relative locations of each sheared *T. brucei* genomic DNA sequence (obtained from the TIGR *T. brucei* genome sequencing project website: www.tigr.org/tdb/mdb/tbdb) and PCR fragments used to compile the complete TbCLH open reading frame are depicted below. Thin lines represent the locations of sheared genomic DNA end sequences and the broken line represents the end sequence of a BAC genomic DNA clone. Accession numbers are shown alongside each sequence. Thick lines represent the locations of the PCR fragments. (B) The β-adaptin/β-arrestin and light chain binding sites of TbCLH are conserved. The upper alignment of human, yeast and trypanosomal clathrin heavy chain shows the high degree of conservation within the β-adaptin and β-arrestin binding site. The residues Q89, F91, K96 and K98 critical for arrestin binding by human clathrin heavy chain are conserved. The lower alignment shows the conservation of residues involved in light chain binding. Large arrows indicate residues involved in trimerisation and light chain binding; small arrows indicate residues preferentially involved in light chain binding. Identical residues are boxed, conservative mutations are shaded. (C) Schematic representation of the domain structure of human β1-adaptin and TbAPβ1. Higher eukaryotic β-adaptins are comprised of a 60-70 kDa trunk domain separated from a 25-30 kDa appendage domain by a ~100 residue linker. The appendage domain is absent in the TbAPβ1 sequence. The binding sites for arrestins, clathrin heavy chain (CHC), AP180, epsin and eps15 in higher eukaryotic β-adaptin are illustrated. Also shown is a putative clathrin binding box sequence in TbAPβ1. Trypanosomal and yeast β1-adaptins terminate with a sequence similar to clathrin binding sequences located in the hinge region of human β-adaptins (HuAPβ1,2,3) and the C-termini of a yeast epsin homologue (ScEnt) and yeast AP180 (ScAP180). The highly conserved leucine and aspartate residues are boxed.

Concanavalin A (ConA) uptake was performed by incubation of cells in serum-free medium supplemented with 5 μg/ml fluorescein ConA (Vector Laboratories, Burlingame, CA) at 4°C for 10 minutes before direct transfer to 37°C for 30 seconds or 1 minute. To follow receptor-mediated endocytosis, 10^7 BSF parasites/ml were incubated in serum-free media containing 50 ug/ml transferrin-Texas Red conjugate (Molecular Probes Inc., Eugene, OR) at 37°C for 30 minutes. For Golgi complex visualisation, BODIPY FL ceramide (Molecular Probes Inc.) was coupled to de-fatted bovine serum albumin (BSA) according to the manufacturer's instructions. Cells were incubated with the conjugated probe at 37°C before back-extracting twice with serum-free medium supplemented with 1.8% de-fatted BSA. All cells were DNA stained with DAPI at 0.5 μg/ml. Cells were examined using a Nikon Microphot II epifluorescence microscope equipped with a CH350 Slow Scan CCD camera (Photometrics, Tuscon, AZ). Digital images were captured using IP Lab spectrum 3.1 (Scanalytics Inc., Fairfax, VA) software or with a Laser Scanning Microscope 510 (Zeiss, Oberkocken, Germany) and processed for printing using Adobe PhotoShop (Adobe Systems Inc., San Jose, CA).

Western blots

1×10^7 cells per lane were electrophoresed on 12% SDS - polyacrylamide gels and blotted onto Hybond ECL nitrocellulose membrane (Amersham life Science Ltd, UK) by wet transfer. Filters were processed as described (Field and Field, 1997).

Yeast expression of HA epitope-tagged TbAPβ1

The full length *TbAPβ1* gene was amplified by PCR from *T. brucei brucei* genomic DNA using the primers, TbBAD1: 5′ CCAAAT-GCATATGATGGAAGCGGTTCTTCGC 3′ and TbBAD2: 5′ GGGG-AATTCTCATGAAAACAAATCATCCAG 3′. The resulting 2.1 kb fragment was subcloned into the *Nde*I and *Eco*RI sites of the yeast expression vector pGADT7 (Clontech) in frame with the N-terminal haemagglutinin (HA) epitope tag. The resulting construct, pGADT7βAd, was transformed into the *Saccharomyces cerevisiae* strain GPY418 by the standard lithium acetate procedure. Wild-type yeast were propagated in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone and 2% Bacto-dextrose). Transformed cells were selected on plates containing SD medium lacking leucine (0.67% yeast nitrogen base, 2% dextrose (Clontech) supplemented with 20

μg/ml adenine, arginine, histidine, methionine, tryptophan and uracil, 30 μg/ml isoleucine, lysine and tryosine, 50 μg/ml phenylalanine, 150 μg/ml valine and 200 μg/ml threonine). Protein was extracted from wild-type and transformed yeast strains using the urea/SDS procedure.

Electron microscopy

For conventional electron microscopy, cells were fixed in 4% paraformaldehyde (PFA-250 mM Hepes; pH 7.4) on ice for 10 minutes followed by 8% PFA-Hepes on ice for 10 minutes and then for 50 minutes at room temperature, washed in PBS and postfixed in 1% osmium tetroxide-1.5% potassium ferrocyanide for 60 minutes at room temperature. The cells were washed in water, incubated in 0.5% magnesium-uranyl acetate overnight at 4°C, washed again in water, dehydrated in ethanol and embedded in Epon. Sections were collected onto grids and stained with lead citrate for contrast. For cryosectioning, cells were fixed in PFA as above, then frozen in 2.1 M sucrose and stored under liquid nitrogen. Thin sections of frozen cells were cut on a Reichert Ultra S microtome with FCS attachment. The sections were then incubated with the clathrin antibody, which was visualised using protein A-gold 6 nm conjugates. All sections were examined using an Omega 912 electron microscope (Zeiss).

RESULTS

Identification of the trypanosomal clathrin heavy chain and β1-adaptin genes

By searching various *T. brucei* sequence databases, we identified partial sequences for numerous potential components of the trypanosomal clathrin-mediated transport system and associated adaptin complexes. These include sequences for two β, μ and σ adaptins, and one each of γ and δ adaptin (see Table 1). Blast analysis and alignments of the retrieved sequences indicate that the African trypanosome has at least two AP complexes. We also retrieved sequence corresponding to clathrin heavy and light chains. Given the depth of coverage of the *T. brucei* genome project sequence pool it is highly probable that the identified gene products represent the total AP complement, although the presence of additional sequences cannot be formally ruled out. Interestingly, the adaptin subunits identified constitute the correct complement to assemble two AP complexes, containing one β, a second heavy chain, and a μ and σ chain each. Homology suggests that these subunits are likely to encode trypanosomal equivalents of the AP-1 and AP-3 complexes.

We decided to characterise the clathrin heavy chain and one of the β-adaptins. We obtained a full length sequence for the open reading frame of 5115 bp of the *T. brucei* clathrin heavy chain gene (GenBank accession no. AJ278858), encoding a protein of 1705 amino acids with a predicted molecular mass of ~191 kDa. TbCLH shares ~34% identity and ~56% similarity with yeast and ~38% identity and ~60% similarity with human clathrin heavy chains, respectively, with similar levels of homology throughout the entire protein (Fig. 1A). The trypanosomal heavy chain is slightly larger due to several small insertions. Whereas the sequence variation of other clathrins is limited to conservative replacements or infrequent gaps of usually less than three residues, TbCLH has a unique insertion of 10 residues in the knee region, between the proximal and distal rod-like domains.

During a screen for components of the trypanosomal secretory pathway we isolated a partial cDNA corresponding to an EST fragment (GenBank accession No. AI881056) homologous to higher eukaryotic β-adaptins. By screening a λ-FIX library with this fragment, we isolated genomic DNA containing full length coding sequence for *TbAPβ1* (GenBank accession no. AF152173). The *TbAPβ1* ORF is 2088 bp in length and encodes a protein of 696 amino acids with a predicted molecular mass of ~76 kDa. TbAPβ1 exhibits greatest homology (~36% identity, ~57% similarity) to higher eukaryotic β-adaptins of the AP1 and AP2 complexes, and ~31% identity and ~50% similarity to yeast β-adaptins.

To determine the closest homologues of TbCLH and TbAPβ1, phylogenetic reconstruction was performed using PAUP (phylogenetic analysis using parsimony; Swofford, 1998). By this analysis, TbCLH is most similar to yeast and plant clathrin heavy chains (Fig. 2A), consistent with current

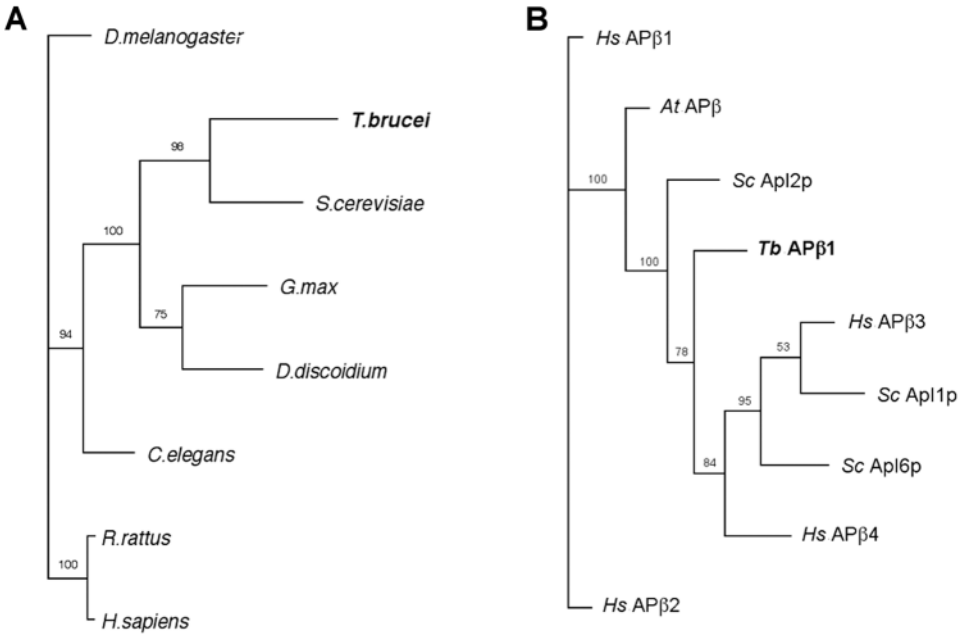

Fig. 2. Conservation of the clathrin and adaptin from *T. brucei*. The evolutionary relationship of TbCLH and TbAPβ1 was analysed using PAUP. TbCLH is demonstrated to be most closely related to the heavy chain of *S. cerevisiae* (A) and TbAPβ1 is most homologous to *S. cerevisiae* β1-adaptin (B). Numbers represent the bootstrap percent confidence for various branch points and horizontal distances represent relative genetic distance. Vertical distances are for clarity only. The β-adaptin sequences used are: Human (*Hs*) APβ1, β2, β3, β4; Yeast (*Sc*) APβ1 (apl2p), β2 (apl1p), β3 (apl6p); *A. thaliana (At* APβ); and *T. brucei* β1 (*Tb* APβ1).

Table 1. Potential trypanosomal adaptor protein homologues

AP	GSS for potential trypanosomal homologues
α-adaptin	None
β1-adaptin	GATSS19TFC, GATTA95TF, GATMA03TF, GATGL57TV, GATBW80TV, GATUF10TF
β-adaptin	GATPS86TR
γ-adaptin	GATLR30TF
δ-adaptin	GATNV22TR, GATMY80TR
μ1-adaptin	GATPY82TF, GATMG07TF, GATTK95TR, GATOM73TR
μ2-adaptin	GATPS24TR, GATBT68TJ, GATGH51TV
σ1-adaptin	GATLE56TR, GATY61TR
σ2-adaptin	GATAC40TPB

The TIGR *T. brucei* genome sequencing project website (www.tigr.org/tdb/mdb/tbdb) was searched using *S. cerevisiae* adaptor protein homologues. Where corresponding sequences were identified it was possible to ascribe sequences to separate AP complexes.

views of eukaryotic evolution. The TbAPβ1 is most similar to yeast APβ1 (Apl2p) (Fig. 2B), suggesting involvement in transport from the TGN, but bootstrap values indicate that an assignment to any one adaptor complex cannot be made based on sequence homology alone. Southern blot analysis demonstrates that both *TbCLH* and *TbAPβ1* are present as single copy genes per haploid genome (data not shown).

Amino acid sequence features of trypanosomal clathrin heavy chain and β1-adaptin

The binding site for β-arrestin has been localised to the first 100 residues of higher eukaryotic clathrin heavy chain (Goodman et al., 1997) and site-directed mutagenesis implicates Q89, F91, K96 and K98 as critical residues. This domain in TbCLH is well conserved and the residues instrumental in β-arrestin binding are found to be invariant (Fig. 1B). Recently, clathrin was shown to interact with β-adaptins through the same site that binds β-arrestins, a groove in the side of the terminal domain of the β-propeller (ter Harr et al., 1998; ter Harr et al., 2000). Although the residues for adaptin binding are conserved, molecular modelling of TbCLH on the crystal structure for the N-terminal domain of rat clathrin heavy chain predicts that the trypanosome protein will have a structure almost indistinguishable from the mammalian protein. This analysis also demonstrates that one of the insertions in TbCLH loops out into this binding groove suggesting there may be a small difference in interactions at this site, the significance of which is unclear at present (data not shown).

On the basis of in vitro mutagenesis analysis, it has been suggested that the light chain binding region extends into the trimerisation domain (Pishvaee et al., 1997), which is predicted to form an α-helix (ter Harr et al., 1998). In this model, residues that effect light chain binding without exhibiting strong effects on trimerisation fall along one face of the helix, enabling the light chain to regulate trimerisation and self-assembly (Pishvaee and Payne, 1998). Analysis of the TbCLH sequence supports this hypothesis, as these residues are highly conserved (Fig. 1B).

The TbAPβ1 sequence lacks the C-terminal globular appendage, or ear, present in higher eukaryotic β-adaptins (Fig. 1C). The appendage domain, which is also absent in yeast β-adaptins, is involved in binding accessory proteins in higher

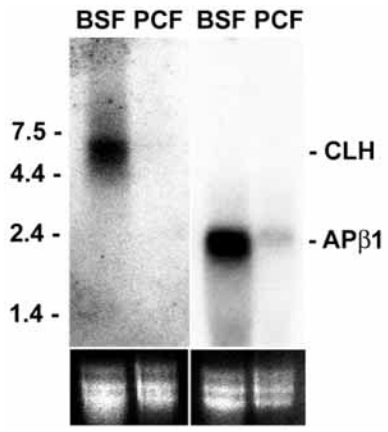

Fig. 3. Trypanosome clathrin and adaptin mRNAs developmentally regulated. Northern blot analysis of *TbCLH* and *TbAPβ1* mRNAs. Total RNA from BSF and PCF cells was hybridised at high stringency with a trypanosomal clathrin heavy chain or β1-adaptin specific probe. Scale at the left represents relative size in kb; ethidium bromide stain of the ribosomal RNA region at the bottom indicates equivalence of loading.

eukaryotes (Owen et al., 2000). As shown in Fig. 1C, both the trypanosomal and yeast β1-adaptin (Apl2) terminate with sequences similar to the canonical clathrin binding box motif (LLpL–, where L denotes leucine, p, a polar residue and –, a negatively charged residue) found in β-arrestin, amphiphysin and the hinge region of other β-adaptins (Dell'Angelica et al., 1998; Kirchhausen, 2000). This sequence is also reminiscent of conserved C-terminal LIDΦ clathrin binding sequences (Φ denotes a hydrophobic residue) in yeast epsin and AP180 proteins (Wendland et al., 1999). Hence, the trypanosome appears to use the same set of peptide signals as higher eukaryotes, indicative of an ancient origin for this system.

The trypanosomal clathrin heavy chain is developmentally regulated

Northern blot analysis of RNA from PCF and BSFs indicate that the *TbCLH* mRNA is present as a single transcript of ~6 kb, and is at least ten times more abundant in the BSF relative to PCF (Fig. 3). The *TbAPβ1* mRNA is present as a transcript of ~2.2 kb and is approximately five times more abundant in the BSF relative to the PCF (Fig. 3), suggesting a more moderate level of developmental regulation than *TbCLH* mRNA.

To characterise the TbCLH and TbAPβ1 proteins, we produced affinity purified rabbit polyclonal antibodies. Anti-TbCLH serum was generated by expressing a portion of the distal leg and trimerisation domain in *Escherichia coli*, which was purified and used as immunogen. The purified antibodies exhibited strong reactivity against the recombinant antigen in induced *E. coli* lysates (Fig. 4A, left). To obtain antibodies against TbAPβ1, rabbits were immunised with a 15 amino acid oligopeptide (residues 568-582) corresponding to a sequence near the C-terminus of the predicted rod domain. We chose to use the peptide route as several attempts to express TbAPβ1 in *E. coli* were unsuccessful, and this region of the protein is predicted to be the least conserved between β-adaptin family members. The specificity of the anti-peptide antibodies was confirmed by probing *S. cerevisiae* that expressed HA-epitope-

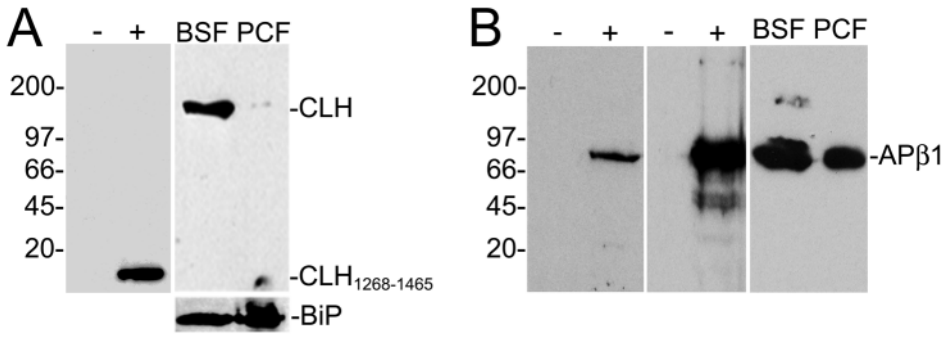

Fig. 4. Developmental regulation of trypanosomal clathrin and adaptin expression at the protein level. Western blot analysis demonstrates developmental regulation of TbCLH protein. (A) Antibodies generated against a fragment of TbCLH (residues 1268-1465) exhibit specific reactivity against the protein, as demonstrated by immunoprobing *E. coli* lysate expressing (+) or not expressing (–) a fragment of TbCLH (left). Immunoprobing of 1×10^7 parasites with anti-TbCLH antibodies demonstrates that the heavy chain is expressed at much higher levels in BSF than PCF cells (right). Trypanosomal binding protein (BiP) is used as a loading control. (B) Reactivity of the anti-peptide antibodies to TbAPβ1 was confirmed by immunoprobing yeast expressing TbAPβ1. Equal lysates (~2×10^7 cell equivalents) from untransformed or transformed *S. cerevisiae* strain GPY418 with pGADT7βAd were loaded per lane. The left and middle panels show yeast expressing (+) or not expressing (–) HA epitope-tagged TbAPβ1 probed with anti-TbAPβ1 antibody (left) or anti-HA antibody (middle). Immunoprobing trypanosomal BSF or PCF lysate with anti-TbAPβ1 antibodies demonstrate approximate equivalence in protein levels (right). Scale at the left represents molecular mass in kDa (A,B).

tagged TbAPβ1, where a band of ~76 kDa was detected, corresponding to the predicted molecular weight of TbAPβ1 (Fig. 4B, middle, left). No reactivity with anti-TbAPβ1 antibodies was observed in lysates from yeast not expressing HA-TbAPβ1.

Northern data suggest a significant degree of developmental regulation for both TbAPβ1 and TbCLH, but due to the post-transcriptional nature of control of gene expression in kinetoplastids, mRNA abundance is not always an accurate reflection of relative protein levels. Hence, to determine the expression level of TbCLH, we probed BSF and PCF cell lysates (Fig. 4A, right). The antiserum identified a single protein of ~190 kDa, highly expressed in BSF cells but barely detectable in PCF. Specificity was confirmed by pre-incubation of the antibody with recombinant CLH, which ablated immunoreactivity on the blot (data not shown). Equivalence of loading was confirmed by reprobing the filter with antibody against *T. brucei* BiP (Bangs et al., 1993), which is expressed about threefold more in BSF compared with PCF. TbAPβ1 affinity purified antibodies were also used to probe BSF and PCF lysates (Fig. 4B, right). This analysis demonstrated a near equivalent level of TbAPβ1 expression because a single band of the appropriate molecular mass (76 kDa) was detectable in both life stages with

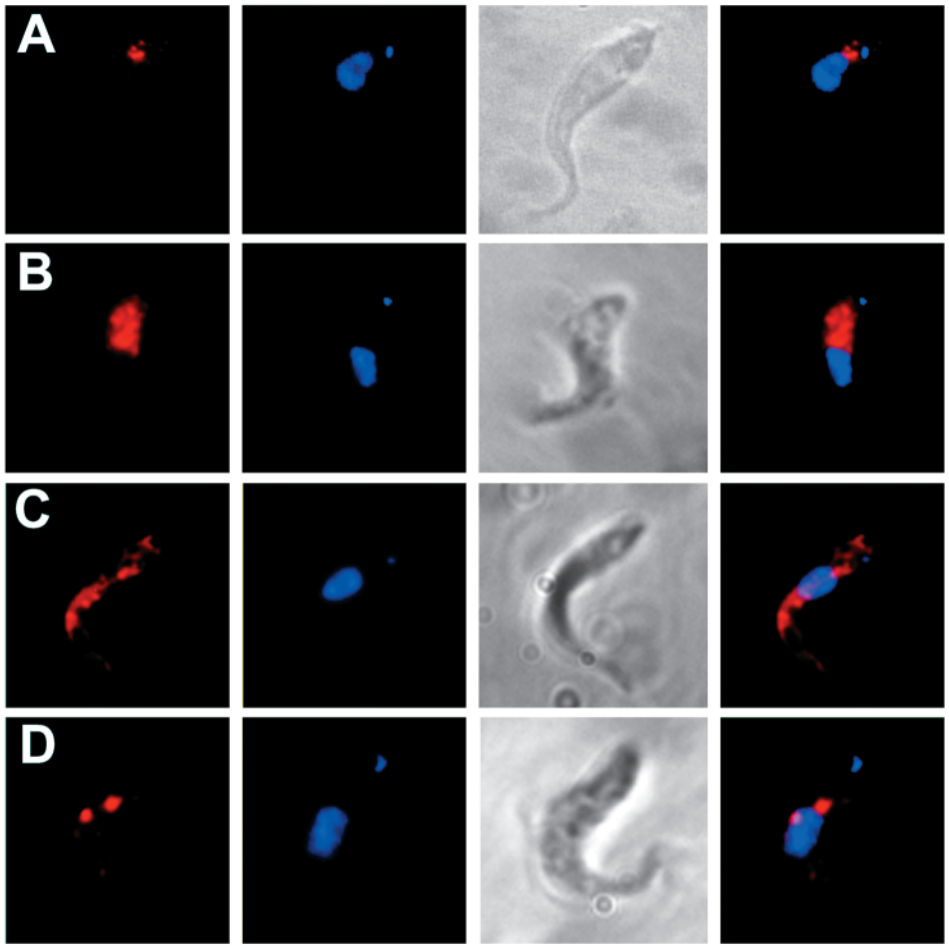

Fig. 5. Localisation of TbCLH and TbAPβ1 in different life stages. The far-left panels show PCF cells (A,C) or BSF cells (B,D) probed with anti-TbCLH (A,B) or anti-TbAPβ1 antibodies (C,D). The centre-left panels show DAPI staining, and the centre-right panels show phase-contrast of the cells. The position of the antibody labelling relative to the nucleus and the kinetoplast is shown in the merged panels (far-right). In PCF, TbCLH is localised to discrete punctata between the nucleus and the kinetoplast but is widely distributed on large membraneous structures throughout the posterior end of the BSF. In the PCF, TbAPβ1 is present on several small peri-nuclear vesicles and reticular elements (C) but, by contrast, is concentrated in two large perinuclear structures in the BSF (D).

approximately equal intensity, indicating that this protein is not subject to significant stage regulated expression. Constitutive TbAPβ1 expression contrasts with the developmental regulation of TbCLH, suggesting that, in *T. brucei*, not all clathrin-mediated transport steps are under developmental control.

Localisation of trypanosomal clathrin heavy chain and β1-adaptin

To determine the subcellular localisations of TbCLH and TbAPβ1 the affinity purified antibodies were used in immunofluorescence analysis of BSF and PCF parasites following paraformaldehyde fixation (Fig. 5). The location of the nucleus and the kinetoplast (mitochondrial DNA) were revealed by DAPI staining. In PCF cells, the clathrin heavy chain is localised between the kinetoplast and the nucleus (Fig. 5A). This localisation is typical of flagellar pocket, endocytic and Golgi structures (Field et al., 2000). In the BSF, TbCLH is extensively distributed throughout the posterior of the cell and is present on numerous large vesicular or tubular structures (Fig. 5B), confirming the extensive upregulation observed by northern and western analysis.

In PCF cells, TbAPβ1 is largely localised to perinuclear vesicles with a lesser amount being diffusely distributed in tubular structures throughout the cell (Fig. 5C). When BSF cells were examined, the distribution of TbAPβ1 was found to be dramatically different to that observed in the PCF and was predominantly localised to two large perinuclear structures in the posterior of the cell between the kinetoplast and the nucleus (Fig. 5D). The near equivalent level of TbAPβ1 immunofluorescence signals in the two life stages is consistent with the western data.

The Golgi complex plays a vital role in clathrin/adaptin-mediated anteriograde transport steps in higher eukaryotes, we therefore investigated the association of trypanosomal clathrin and adaptin protein with the membranes of this organelle. We used BODIPY ceramide to label the Golgi stacks (Field et al., 1998; Field et al., 2000) and observed that TbCLH was localised to elements juxtaposed to the Golgi complex in BSFs (Fig. 6A). Significantly, no complete colocalisation was observed. Association of clathrin with vesicular and tubular elements, close to the Golgi complex was confirmed at the ultrastructural level by cryo-immunomicroscopy (see below). Counter-staining BSF cells with BODIPY ceramide and anti-TbAPβ1 demonstrated that the more anterior TbAPβ1-positive structure partially localises to the Golgi complex, most probably the trans face (Fig. 6B). Association of TbAPβ1 with the trans-Golgi complex and the virtual absence of this protein from the region of the flagellar pocket suggests that TbAPβ1 is probably an AP1β-adaptin orthologue. However, the differential distribution of the protein in BSF and PCF suggests that TbAPβ1 could be involved in more than one transport system. Co-staining PCF and BSF cells for TbCLH and TbAPβ1 demonstrated only partial colocalisation of these two proteins (Fig. 6C,D). Failure to detect significant colocalisation for clathrin and TbAPβ1 in the BSF is most likely a consequence of the large amount of clathrin involved in mediating the massive levels of endocytosis relative to a far smaller population of clathrin molecules associated with the Golgi, and localising with TbAPβ1.

By DAPI staining of the kinetoplast and nucleus, it is possible to position cells at particular points during the cell cycle (Woodward and Gull, 1990). The distribution of TbCLH

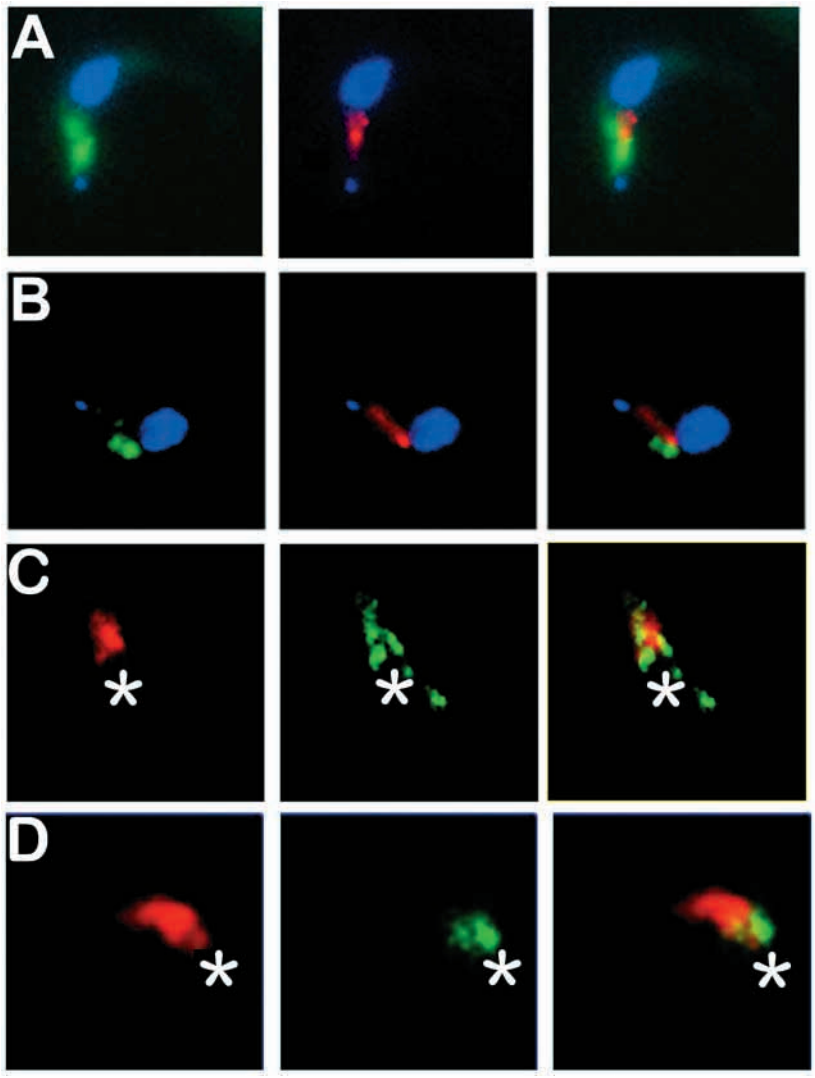

Fig. 6. TbCLH and TbAPβ1 closely associate with the Golgi complex, but are on distinct membranes. (A,B) BSF cells were stained with TbCLH (A, left) or TbAPβ1 (B, left), BODIPY-ceramide (middle), and DAPI (in all panels); the merged image is shown on the right. TbCLH is observed in structures juxtaposed to the Golgi complex, whereas the TbAPβ1 membranous structure nearest to the nucleus partially co-localises to the Golgi complex as seen in yellow in the merged image. (C,D) PCF (C) and BSF (D) cells labelled with rabbit anti-TbCLH (red) and mouse anti-TbAPβ1 (green) and examined by confocal microscopy. The merged image is shown on the right; the asterix indicates the position of the nucleus. In neither of these life stages is there significant coincidence.

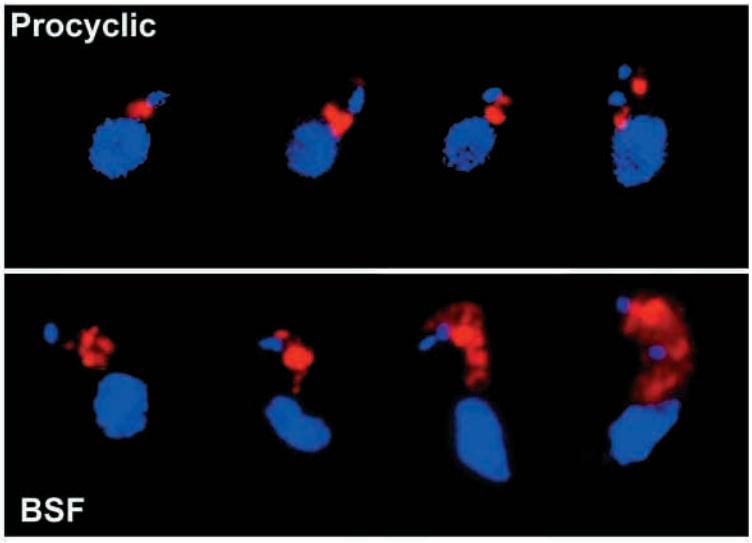

Fig. 7. Differential localisation of TbCLH during the cell cycle. TbCLH is closely associated with the kinetoplast in the PCF and as the kinetoplast divides during mitosis, the pool of TbCLH is also observed to divide and separate with this structure. This behaviour is consistent with the vast majority of TbCLH being associated with membranes subtending the flagellar pocket. The distribution of TbCLH during the cell cycle in BSF is also shown; again TbCLH infills following kinetoplast division, but is less clear cut due to the extensive clathrin network in this life stage.

during the cell cycle of PCF and BSF cells is shown in Fig. 7. Overall expression levels of the protein do not alter significantly and the major features of individual structures remain, suggesting that there is no morphological alteration of the clathrin apparatus during mitosis. Throughout the PCF cell cycle TbCLH remains tightly associated with the kinetoplast but most significantly, when the kinetoplast initiates division, the TbCLH structures are also seen to divide into two pools, both of which remain juxtaposed to the kinetoplast (Fig. 7). This behaviour is consistent with the highly coordinated fashion in which *T. brucei* replicates organelles, including those of the endomembrane system and the Golgi complex (Field et al., 1998; Field et al., 2000).

Clathrin heavy chain is associated with endocytic vesicles

To demonstrate directly that TbCLH is involved in endocytosis in *T. brucei*, ConA was used as a marker for membrane bound ligands at the flagellar pocket and endocytic vesicles (Brickman et al., 1995). When bloodstream form parasites incubated with fluorescently labelled ConA at 4°C were warmed to 37°C for 30 seconds, most ConA is confined to the flagellar pocket, with a lesser amount present in adjacent vesicular structures (Fig. 8A). Under these conditions, there is little co-localisation of ConA with TbCLH, which underlies the flagellar pocket. Following a 1 minute incubation at 37°C, a greater fraction of ConA has internalised and there is increased co-localisation with TbCLH (Fig. 8B). To demonstrate more precisely the involvement of TbCLH in the process of receptor-mediated endocytosis, we analysed uptake of fluorescent transferrin, which is endocytosed by a GPI-anchored heterodimeric receptor complex, ESAG6/7 (Steverding et al., 1994). In this case we observed almost complete colocalisation of transferrin with TbCLH (Fig. 8C). We conclude that TbCLH is indeed a component of endocytic vesicles and the greatest concentration of TbCLH is not on the flagellar pocket membrane but vesicular and tubular structures underlying it. Significantly, the TbCLH-containing structures receive endocytic traffic rapidly and hence represent true clathrin-coated vesicles. Interestingly, the presence of transferrin in clathrin-containing structures suggests that, in trypanosomes, GPI-anchored proteins can be endocytosed by a clathrin-dependent mechanism.

Ultrastructural localisation of TbCLH

Previous workers have reported the presence of vesicular coats on membrane structures associated with the flagellar pocket region that have a clear similarity to the clathrin coats of higher eukaryotes (Webster and Shapiro, 1990). Analysis of PFA fixed Epon-embedded BSF cells demonstrated the presence of clathrin-like coated vesicles and collecting tubules in the vicinity of the flagellar pocket indicated by the presence of an

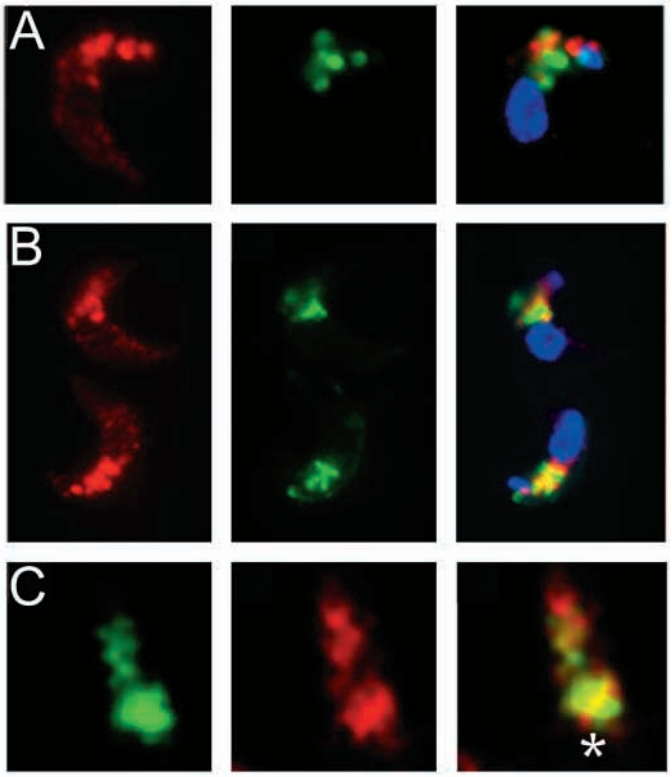

Fig. 8. ConA and transferrin uptake demonstrate TbCLH is involved in endocytosis. BSF cells were pulsed with fluorescein-ConA for 30 seconds (A) or 1 minute (B), or with Texas Red-transferrin (C). The localisation of TbCLH is shown in the left panel, ConA or transferrin in the middle panel, and the merged image in the right panel. The position of the nucleus is shown by DAPI staining (blue) or marked by an asterix in C. After 1 minute, there is strong co-localisation between the lectin and TbCLH. There is a high degree of transferrin/TbCLH co-localisation, indicating that clathrin is present on structures receiving cargo targeted to endosomes by receptor-mediated endocytosis.

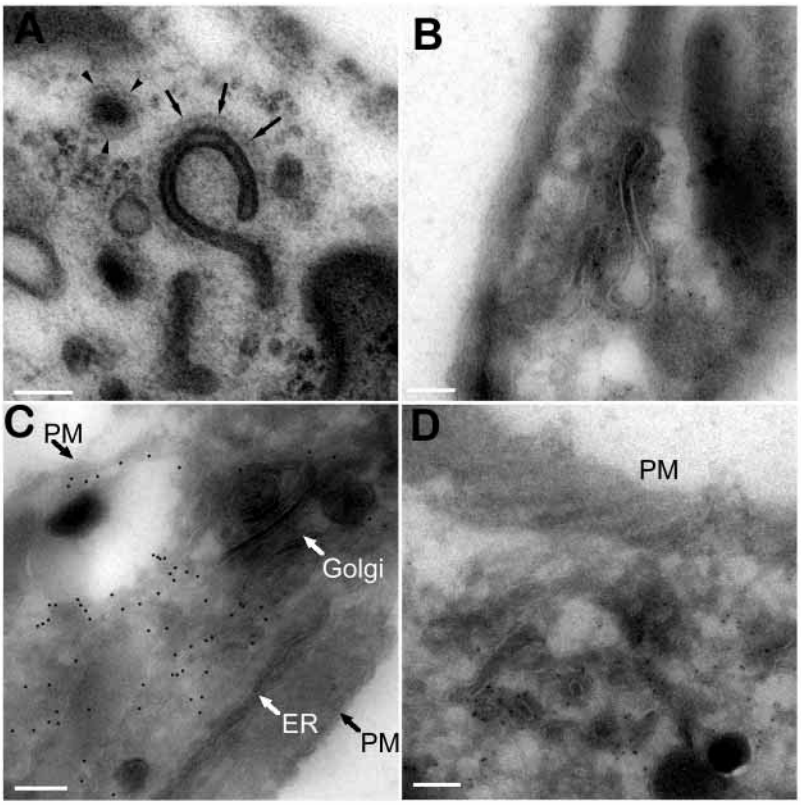

Fig. 9. Ultra thin cryosections showing subcellular localisation of TbCLH. (A) Conventional Epon section showing clathrin-like coats on a vesicle (arrowheads) and a tubular profile (arrows). (B-D) Cryosections labelled with TbCLH followed by 6 nm protein A gold show clathrin in association with a tubular profile (B); on membranes and vesicles in a region between the Golgi and the flagellar pocket (C); and also TbCLH labelling of membranes and vesicles just below the plasma membrane (D). In C, the 6 nm gold particles originally used for increased resolution have been digitally enhanced using Adobe Illustrator by placing black circles corresponding to ~15nm diameter over the Gold particles. Bars, 100 nm, the position of the Golgi complex, endoplasmic reticulum (ER) and plasma membrane (PM) are indicated.

electron dense fibrillar coat (Fig. 9A). To further refine the localisation of TbCLH and to determine if these structures do indeed represent trypanosome clathrin, cryo-immunogold electron microscopy was performed using the TbCLH antibodies. The location of the gold particles confirmed that clathrin is indeed a component of the coat on the collecting tubules (Fig. 9B). In addition, TbCLH immunoreactivity is widely distributed amongst membrane structures in the posterior region of the cell, as demonstrated by the digitally enhanced gold particles shown in Fig. 9C. A minority of TbCLH is also associated with membranes associated with the trans-Golgi face (Fig. 9C) and coated vesicles (Fig. 9D).

DISCUSSION

The existence of clathrin-mediated endocytosis in trypanosomatid parasites has been suggested previously by numerous ultrastructural and biochemical analysis. This is of wide significance, as the vast majority of cell surface proteins in *T. brucei* are GPI-anchored and evidence suggests that lipid-anchored proteins may use clathrin-independent endocytic pathways. VSG protein has been observed in coated vesicles (100-150 nm diameter) and also in structures derived from the flagellar pocket (Webster and Shapiro, 1990). Coated vesicles have been isolated by cell fractionation and, significantly, the clathrin-like coat structures derived from BSF parasites were absent from equivalent vesicles obtained from the PCF stage of *T. brucei* and insect stages of *Leishmania* (Shapiro and Webster, 1989). Since the uptake of both fluid phase markers and other markers is much more efficient in the BSF than the PCF (Langreth and Balber, 1975), regulation of the clathrin-like coat components represents one possible mechanism for mediating the high levels of endocytosis observed in these parasites (Overath et al., 1997). Conversely, the absence of the clathrin-like coat in the PCF, in which receptor mediated endocytosis has recently been demonstrated to occur (Lui et al., 2000), may explain the much reduced endocytic rate in this life stage. Our data are in full agreement with this model and, furthermore, provide molecular evidence that the coat of trypanosome endocytic vesicles contains the clathrin heavy chain.

TbCLH is extremely highly conserved, as has been observed for other heavy chain sequences from yeast to humans, but the evolutionary distance between trypanosomes and higher eukaryotes is rather more significant. The high pressure to preserve the primary structure of the heavy chain is probably a reflection of the highly extended inter leg contacts required for assembly of the triskelions and the large number of interactions also required for cargo binding and the regulation of coat formation (Kirchhausen, 2000). The N-terminal domain of TbAPβ1 exhibits significant similarity to higher eukaryotic adaptins and is most homologous to yeast β1-adaptin. However, the appendage domain is absent indicating it is possible to efficiently form clathrin cages without this specialised binding platform for accessory proteins. TbAPβ1 is found to terminate with a sequence similar to the clathrin binding box motif.

Several components of the endo-membrane system of *T. brucei*, such as BiP and p67, are subject to a degree of developmental regulation, being more highly expressed in the BSF than the PCF (Bangs, 1998). The observation that TbCLH is subject to strong upregulation in the BSF, whereas the Golgi-associated TbAPβ1 is expressed at more equivalent levels, is consistent with the elevated endocytosis and recycling in the BSF. A high rate of endocytosis at the flagellar pocket is proposed to be the result of dependence on host nutrients, but may also play a vital role in immune evasion. Nutrient acquisition via receptor-mediated endocytosis is presumably also required in the PCF, but only the BSF has to face the mammalian host immune system (Overath et al., 1997; O'Beirne et al., 1998), and this may be a primary reason for elevated endocytic levels in BSFs.

Both TbCLH- and TbAPβ1-containing compartments

exhibit dramatic morphological restructuring between life stages. In the PCF, TbCLH has a limited distribution, localising predominantly to the flagellar pocket, whereas in the BSF it is extensively distributed to large and numerous tubulo-vesicular structures throughout the cytoplasm. In the BSF, the trypanosomal clathrin heavy chain is involved in endocytosis at the flagellar pocket and is present on coated vesicles near to the TGN. We cannot rule out the possibility that the more limited PCF distribution is due, at least in part, to threshold effects, but the presence of significantly more bright foci in BSF immunofluorescence images compared with the PCF suggests that the BSF does indeed contain a more elaborate set of clathrin compartments.

The endocytic system of BSF trypanosomes has a characteristic morphology consisting not only of discrete vesicles but also of stacks of collecting tubules (Langreth and Balber, 1975; Brickman et al., 1995; Jeffries et al., 2001). By cryoimmuno-EM, the clathrin heavy chain is shown to be present on coated vesicles and highly extended tubules adjacent to the flagellar pocket, as well as structures associated with the TGN. These tubules have been proposed to be analogous to tubular endosomes and are the site of endocytosed material trapped at low temperatures (Brickman et. al., 1995). Clathrin has previously been found in peripheral endosomes (Sorkina et al., 1999), where it may be associated with AP-3. Whether the clathrin-coated tubules identified in this study are part of the endocytic apparatus or the endosomal network remains to be elucidated

The distribution of TbAPβ1 in PCF cells is observed to be atypical of previously studied β-adaptins. In the PCF it is concentrated in several small perinuclear vesicles and to a lesser degree in reticular structures reminiscent of the ER (Bangs et al., 1993). In the BSF, TbAPβ1 is redistributed to two large membranous structures adjacent to the nucleus on the posterior side and there is reduced visible reticular staining. The structure nearest to the nucleus partially localises to the Golgi complex. There is only partial colocalisation of TbCLH and TbAPβ1, suggesting that adaptin is not associated with the majority of the clathrin involved in endocytosis and is most likely associated with the TGN. This localisation and phylogenetic data indicate that the adaptin studied here is most likely to be a component of the AP-1 complex. In mammals, AP-1 has been localised to early/recycling endosomes (Futter et al., 1998), which may account for some of the peripheral staining observed with the anti-TbAPβ1 antibodies. However, we cannot formally rule out the possibility that TbAPβ1 is a component of the TGN-associated AP3 complex. The trypanosomal database contains partial sequences for at least one other trypanosomal β-adaptin and sufficient AP subunits to form two distinct full AP complexes. It is noteworthy that it is not possible to clearly define sequences for three adaptor complexes as observed in other eukaryotes and there are no sequences for the large α subunit of the AP2 complex currently in the databases.

Phylogenetic analysis of major components of the eukaryotic heterotetrameric transport coat proteins indicates that they have evolved through gene duplication of a common heterodimer ancestor (Schledzewski et al., 1999). Gene duplication has also been demonstrated to be a mechanism for elaboration of other components of the secretory pathway (Field and Field, 1997). The most recent gene duplication of the heterotetrameric transport coat proteins was that giving rise to the AP1 and AP2 complexes and from current *T. brucei* genome sequence data, only two AP complexes are discernible in this divergent eukaryote. Further work is required to assign the *T. brucei* AP complexes as orthologues of the mammalian complexes.

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